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Monitoring human risk and exposure to trinitrotoluene (TNT) using haemoglobin adducts as biomarkers

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Abstract

Two studies were carried out in a trinitrotoluene (TNT) plant using TNT haemoglobin (TNT-Hb) adduct as a biomarker to study dose-adduct and adduct-response relationships. In the first study, TNT-Hb adduct levels were determined in 117 TNT-exposed workers in different working sites with different exposure conditions. External exposure was calculated from the inhaled air concentration plus skin contamination. TNT-Hb adduct levels in blood were significantly correlated with their external exposure to TNT. Two methods, HPLC-UV and CI-ELISA, were developed for measuring TNT-Hb adduct: good correlations ($r = 0.77$ and 0.86) were found between these 2 methods. In the second study, TNT cataract was used as an indicator of health effects. The prevalence of cataract and the degree of lenticular damage increased with the increase of blood TNT-Hb level.

Keywords: Trinitrotoluene; Hb adduct; Biomarkers; Monitoring

1. Introduction

2,4,6-Trinitrotoluene (TNT) is a commonly used explosive since World War 1. Exposure to TNT has been reported to result in serious toxic effects, such as liver injury and marked changes in the haemopoietic system producing anaemia [1,2]. In China, chronic occupational exposure was shown to cause mainly hepatomegalia and cataract. The incidence of hepatomegalia was 41.27% and that of cataract was 78.57% in the TNT workers with prolonged exposure, whereas anaemia was rarely found [3]. Hepatitis-like symptoms and signs were not readily differentiated from hepatitis of viral origin [4]. Biomarkers that reflect early cellular response after exposure, provide a new strategy for exposure assessment.

Many techniques have become available for the detection of human exposure to carcinogens using either DNA or Hb adducts as dosimeters [5–8]. Based on these experiences, we explored the possibility of using a biomarker for monitoring TNT exposure in humans. Previous animal studies have demonstrated that TNT administration results in the formation of covalent binding between TNT and macromolecular proteins including serum albumin, Hb and hepatic and renal proteins [9]. The Hb adduct was dose dependent

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and more stable than that formed in plasma, liver and kidney. 4-Amino-2,6-dinitrotoluene (4A) and 2-amino-4,6-dinitrotoluene (2A) were recovered by solvent extraction as major products of acid hydrolysis of rat blood using mass spectrometry and HPLC co-chromatography. However, only 4A was found in exposed human blood samples [10]. Based on data of in vitro and in vivo studies, it was postulated that TNT undergoes rapid reduction to hydroxylamine, followed by bioactivation to form covalent adducts. Two methods, HPLC [11] and CI-ELISA [12] have been developed for measuring human TNT-Hb adduct and the results of CI-ELISA and HPLC analysis showed a good correlation ($r = 0.86$) [13].

2. Materials and methods

2.1. Determination of external exposure

Thirty-seven workers at each site were investigated. External exposures to TNT were calculated from the inhaled TNT amount plus skin contamination.

Inhaled TNT. Subjects were asked to carry personal air samplers (P 2500B, Dupont) to collect air samples from the breathing zone at a flow rate of 1 l/min for 8 h. Intake was calculated as the amount of TNT inhaled assuming a respiratory volume of 20 l/min.

Skin contamination. Samples of the same subjects were obtained according to Proportional Areas Method [14]. Fixed areas of 9 different parts of the worker's body (forehead, neck, upper arms, hands, back, abdomen, thighs, shanks and feet) were smeared with cotton swabs soaked with ethanol. TNT was determined and adjusted by the ratio of smeared areas to the body surface area (BSA) according to the following formulas:

$$\text{BSA } (\text{cm}^2) = [0.006 \times \text{height (cm)} + 0.0128 \times \text{body weight (kg)} - 0.1529] \times 10^4$$

Total contaminating amount = average contamination × BSA × 0.841

TNT determination. A colorimetric method described by Gronsberg [15] was used to determine TNT. The method depends on collecting and dissolving TNT with alcohol and then the alcoholic solution is treated with sodium hydroxide solution. The violet colour thus obtained is compared with a series of standards.

2.2. Determination of TNT-Hb adduct

Blood samples were collected from 115 workers under different exposure conditions and from 22 referents working at the same plant but not exposed to TNT. All the blood samples were determined by both HPLC and CI-ELISA methods.

Preparation of blood samples. Heparinised blood (2.0 ml) was drawn intravenously and centrifuged at 2000 × *g* for 15 min to separate plasma and red blood cells (RBC). RBC were washed 2–3 times by the addition of 2–3 volumes of isotonic saline and finally centrifuged at 3000 × *g* for 5 min to remove the supernatant. After washing, RBC were lysed by the addition of 2 volumes of ice water, and then vortexed for 5 min. The lysate was centrifuged at 10 000 × *g* for 10 min to remove unbroken cells (debris). The clear supernatant contained Hb. Hb concentration was determined by the cyano-metHb method [16].

HPLC method [11]. Hb solution (1.0 ml) prepared from the above procedures was suspended in equal volume of sodium dodecyl sulfate (SDS), and washed twice with 2 volumes of ether to remove free amines contained in the Hb. Then, 0.2 ml HCl (2 mol/l) was added, and the suspension was shaken for 2 h at 37°C. The hydrolysate was neutralised by $NaHCO_3$ and extracted with 2 ml ether in triplicate. The pooled ether phase was removed under the stream of nitrogen. The residues were dissolved in methanol, and reconstituted to 100 μl with mobile phase. The released amine 4A was determined by HPLC using a Waters 510 equipped with Ultraphere IP C18 (4.6 × 150 mm, 5 μm) preceded a guard column with sample packing (4.6 × 45 mm), a UV detector set at 230 nm. Isolation elution was carried out with methanol/water (48/52) at 1.0 ml/min. An aliquot of the solution (25–50 μl) was injected to HPLC to determine the 4A concentration by external standard of 4A. In this method 0.015 ng/g Hb of TNT-Hb adduct can be detected.

CI-ELISA method [12]. Artificial antigens have been prepared by coupling 4A, which is the major metabolite released from the Hb adduct, to bovine serum albumin (BSA) as antigen and diphtheria-toxid (DT) as immune stimulant. Antibody was elicited by intradermal injection of 4A-DT to rabbits. The titre varies from $1:8 \times 10^5$ to $1:64 \times 10^5$. Polystyrene plates were coated with

Table 1
TNT exposure via different routes

Working type	No.	Exposure (mg)		Total amount (mg)
		Inhaled	Skin	
Loading	4	37.60 ± 29.8	317.8 ± 154.1	355.4 ± 180.9
Grinding	5	4.10 ± 1.7	218.4 ± 121.4	222.5 ± 121.5
Screening	3	28.60 ± 4.2	145.3 ± 90.6	173.9 ± 100.6
Pressing	7	2.70 ± 2.7	125.2 ± 92.6	127.9 ± 91.6
Packing	4	3.60 ± 4.2	66.6 ± 10.2	70.2 ± 70.2

Inhalation vs. total $r = 0.74$.
Skin vs. total $r = 0.99$.

4A-BSA in 0.05 mol sodium carbonate buffer (pH 9.6) at 4°C overnight. Then, the plates were washed with PBS containing 0.1% Tween 4 times. A mixture of 50 μl test blood sample prepared as above with equal volume *f* antibody was added to each test well and incubated at 37°C for 1.5 h. After washing the plates with 0.1% Tween-PBS, 100 μl goat anti-rabbit HRP-IgG were added, and the plates were incubated at 37°C for 1 h followed by washing again. The enzyme reaction was developed by the addition of 100 μl of the substrate solution *o*-phenylenediamine, and was stopped after a 15-min, 37°C incubation by the addition of 25 μl 2.0 M H_2SO_4. Plates were read with an ELISA reader at 490 nm. The linear range of this method is between 0.5 and 1000 ng/ml and 0.05 ng 4A can be detected in each sample. The coefficients of the variation are approximatly 8.0 and 15.0% for intra- and inter-assay, respectively. The recovery of 4A in the Hb was 84–100%.

2.3. Examination and diagnosis of cataract [17]

Since TNT-induced cataracts are progressive and eventually form irreversible or persistent changes in lens, this study has been restricted to the workers who had been exposed to TNT at the same working condition and worked at least more than 1 year since first employment. For ophthalmologic examination, the lens were examined with a slit-lamp. The diagnosis of TNT cataract was

Table 2
The relationship between TNT-Hb adduct and external exposure of TNT

Job	No. of subjects	TNT-Hb levels (ng/g Hb)		TNT exposure (mg)
		HPLC	ELISA	
Loading	12	1105.9 ± 543.7	182.1 ± 79.7	355.4 ± 180.9
Screening	13	748.6 ± 757.1	157.3 ± 113.5	222.5 ± 121.5
Grinding	4	876.9 ± 424.9	152.6 ± 127.7	173.9 ± 100.6
Pressing	13	624.9 ± 302.4	85.7 ± 64.9	127.9 ± 91.6
Packing	42	114.8 ± 124.1	34.1 ± 28.6	70.2 ± 8.5
Repairing	6	71.7 ± 65.4	19.1 ± 30.3	
Control	22	< 26.4	5.8 ± 10.2	
Total	112			

HPLC vs. TNT exposure $r = 0.88$.
ELISA vs. TNT exposure $r = 0.89$.
HPLC vs. ELISA $r = 0.77$.

based on the Diagnostic Criteria and Principles of Management of Occupational Chronic Trinitrotoluene Poisoning [18].

3. Results

3.1. Monitoring human exposure

Since TNT gains access to the body through multiple routes, total TNT exposure was estimated by the combination of inhaled TNT and skin contaminating TNT. Table 1 shows TNT exposure in 5 main working processes (loading, grinding, screening, pressing and packing). It also indicates that the most important route of exposure was skin contamination. Two methods, HPLC and CI-ELISA, were used to measure TNT-Hb adduct. As shown in Table 2, TNT-Hb adduct levels were significantly correlated with external exposure (by HPLC: $r = 0.88$, and by CI-ELISA: $r = 0.89$), the results obtained by the 2 methods also being correlated ($r = 0.77$). Despite a good correlation with those measured by CI-ELISA, adduct levels measured by HPLC after extensive hydrolysis were much higher. With both methods, only trace amount of TNT-Hb adduct was found in the control subjects.

For the lack of raw material in the factory, some workers (shown as Group 2 in Table 3) were irregularly exposed. For example, loading workers in Group 2 were exposed to TNT only every other week. The TNT-Hb adduct levels of these workers were lower (HPLC: 337.1 ± 266.4 ng/g Hb, CI-ELISA: 82.2 ± 65.2 ng/g Hb) than that of the workers in Group 1 with regular exposure (HPLC: 1105.9 ± 543.7 ng/g Hb, CI-ELISA: 182.1 ± 79.7 ng/g Hb). In general, Hb adduct is considered as a stable adduct with relatively long life-span. This was confirmed by this study. In the pressing site, Group 2 workers have not been exposed to TNT for more than 2 months. Although the TNT-Hb adduct levels of workers in this group were lower than that of Group 1, a still significant amount of TNT-Hb adduct was detected (HPLC: 205.3 ± 320.1 ng/g Hb, CI-ELISA 26.1 ± 19.1 ng/g Hb).

3.2. Monitoring of human risk

Cataract was chosen as an indicator for toxic effects of TNT based on the fact that cataract sometimes is the first and only sign of the TNT-exposed workers. Cataract was detected in 29 out of 126 exposed workers, with a prevalence of 23%. Twenty-six were diagnosed as stage one, 3 as stage two and the other 7 as suspected cases. The relationship between the prevalence of TNT cataract and TNT-Hb adduct levels is shown in Fig. 1. The prevalence of lenticular damage seems to increase with the increase of TNT-Hb adduct level. No cataract was found when the TNT-Hb level was below 140 ng/g Hb, even for duration of employment up to 20 years. Three subjects with high TNT-Hb levels (> 1600 ng/g Hb), were all diagnosed as TNT cataract.

4. Discussion

In the past, the determination of the urinary excretion of TNT and its metabolites (2A and 4A)

Table 3
The relationship between TNT-Hb adduct and external exposure condition

Job type	No. of subjects	TNT exposure (mg)	TNT-HB levels (ng/g Hb)	
			HPLC	ELISA
Loading		355.4 ± 180.9		
Group 1	12	Regular exposure	1106 ± 543.7	182.1 ± 79.7
Group 2	20	Exposed every other week	337.1 ± 266.4	82.2 ± 65.2
Pressing		127.9 ± 91.6		
Group 1	13	Regular exposure	624.9 ± 302.4	85.7 ± 64.9
Group 2	5	Away from TNT for more than 2 months	205.3 ± 320.1	26.1 ± 19.1

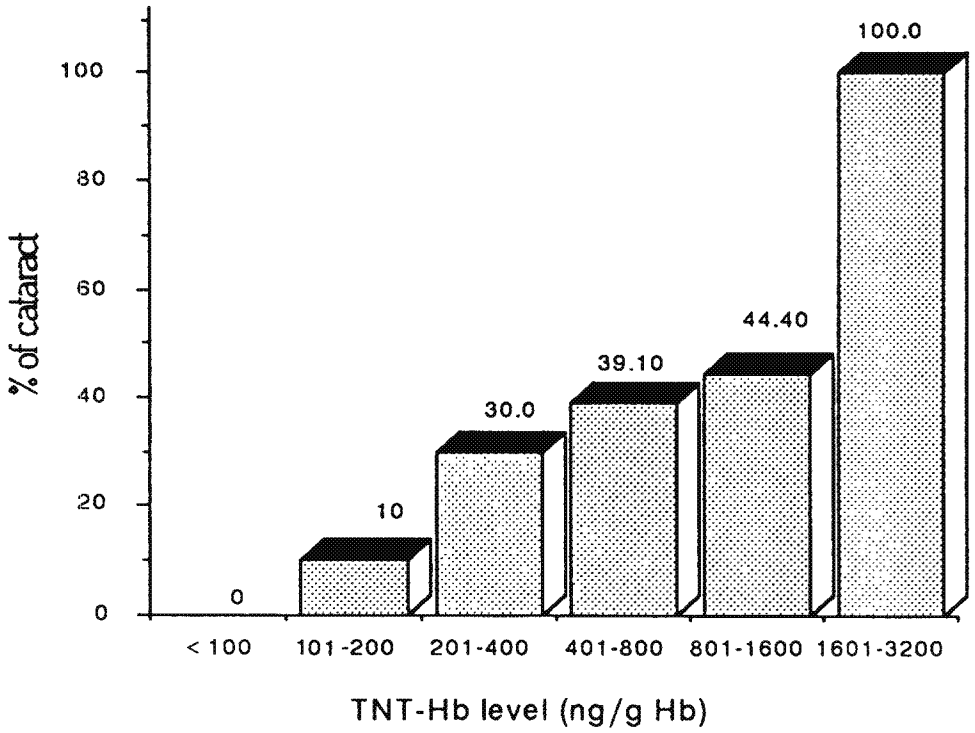

Fig. 1. Relationship between TNT-Hb levels and cataract.

was commonly used to monitor human exposure to TNT. The results were not satisfactory, because of the large interindividual variation and usually nothing could be detected after 2–3 days since the last exposure. Although 4A is the main metabolite both in urine and blood, blood TNT-Hb adduct is more reliable and stable. Fig. 2 shows the metabolic pathway of TNT. 4A in the urine represents the result of detoxification. Its excretion is usually complete within 3–4 days after exposure. Besides, the formation of protein adduct would take into account interindividual differences in the biotransformation of TNT into active metabolites. According to present understanding, macromolecular binding is likely to be correlated with toxic effects. Therefore, the determination of Hb adducts seem to offer distinct advantages over the measurement of TNT metabolites in urine.

DNA adduct is often considered as the critical biomarker for monitoring carcinogen exposure. Although TNT is not classified as a carcinogen, the results of our previous studies confirmed the formation of covalent protein adducts in various rat tissues. The present studies further prove the formation of TNT-Hb adduct in exposed workers and their correlation with exposure levels. Thus, such an approach is not limited to genotoxic carcinogens, but is potentially extensible to a number of organic compounds able to form unique adducts with Hb and other proteins.

Workers from 5 different working sites were exposed to various levels of TNT. TNT-Hb adduct was correlated with external exposure ($r = 0.88$ or 0.89), whereas only trace amounts were found in unexposed workers. Owing to the long life-span of Hb, TNT-Hb adduct can be detected for a long time after exposure and can be used to assess intermittent as well as continuous exposures. In addition, it could reflect intake from multiple routes. For TNT exposure in the present studies, the adduct levels in exposed workers were more related to skin contamination than to air concentration, in-

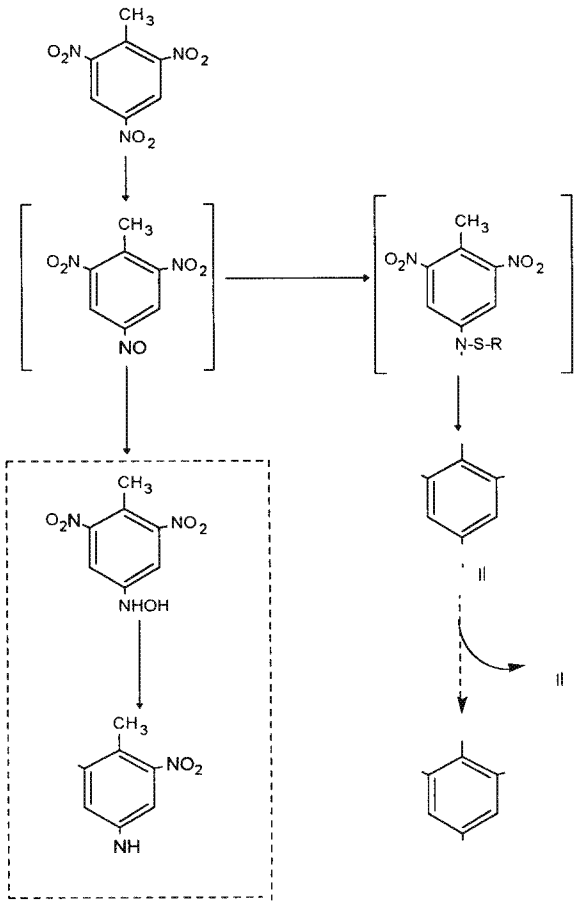

Fig. 2. Metabolite pathway of TNT.

dicating that skin contamination is the main source of internal dose, which could be lowered through simple measures of personal hygiene.

The link between macromolecular binding and biological end points is much more difficult to demonstrate. In this study, cataract was chosen as a relevant end point based on the hypothesis that TNT may bind with the protein of eye lens. The level of eye lens adduct is supposed to be related both to the degree of lenticular damage and to the level of TNT-Hb adduct. The much longer life-span of eye lens protein could explain why TNT cataract is irreversible or persistent. Although the mechanisms leading to TNT cataract are not clear, there is a good correlation between TNT-Hb adduct and cataract.

In summary, TNT-Hb adduct is a useful biomarker and shows the following advantages: (i) Hb is easily obtainable and 1–2 ml blood is enough for analysis; (ii) the life-span of Hb adduct in humans is 18 weeks and therefore the adduct can be detected many weeks after exposure and the formed adduct accumulates during chronic exposure; (iii) the levels of Hb adduct were correlated with exposure levels; (iv) TNT-Hb reflects exposure by multiple routes irrespective of exposure patterns; (v) since target tissues may not be accessible for sampling, Hb adduct can be used as a biomarker for risk assessment.

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